昆虫学报 *Acta Entomologica Sinica*, February 2009, 52(2): 126 – 132 ISSN 0454-6296

Effects of toxin huwentoxin-III from the venom of the Chinese bird spider, *Ornithoctonus huwena* (Araneae: Theraphosidae) on neuronal voltage-gated ion channels in cockroach *Periplaneta americana*

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Abstract: Huwentoxin-III purified from the venom of the Chinese bird spider, *Ornithoctonus huwena* (Wang *et al.*), is an insect neurotoxic peptide. The effects of huwentoxin-III on neuronal voltage-gated ion channels were studied by using whole-cell patch clamp technique. Huwentoxin-III specifically inhibited voltage-gated sodium channels in dorsal unpaired median neurons of adult cockroach *Periplaneta americana* ($IC_{50} \approx 1.106$ μmol/L) while having no evident effect on voltage-gated potassium channels. HWTX-III inhibited insect voltage-gated sodium channels through a novel mechanism distinctive from other spider toxins, did not affect the activation and inactivation kinetics, and not evidently shift the steady-state inactivation curve. Thus, its specificity and novel mechanism on insect neuronal voltage-gated sodium channels make it an interesting tool for investigating the multiple molecular forms of voltage-gated sodium channels and exploiting new and safe insecticides.

Key words: *Ornithoctonus huwena*; huwentoxin-III; *Periplaneta americana*; dorsal unpaired median neurons; sodium channel; whole-cell patch clamp technique

1 INTRODUCTION

Voltage-gated sodium channels (VGSCs) are widely distributed in most excitable tissues, play multiple roles in many important physiological processes. They are composed of a functional pore-forming α subunit (260 kDa) associated with up to four different auxiliary β-subunits (21 – 23 kDa) (Catterall, 2000; Stevens *et al.*, 2001). Similar to the shaker potassium channel, the three-dimensional structure of sodium channel is a bell-shaped molecule (Sato *et al.*, 2001; Sokolova *et al.*, 2001). Up to now over ten mammalian subtypes ($Na_v1.1 – 1.9$ and Na_vx) and three insect VGSCs have been cloned and successfully expressed (Warmke *et al.*, 1997; Goldin *et al.*, 2000). Despite their similarity in electrophysiology, primary structure and allocation of functional domains, the pharmacological properties of insect VGSCs distinguish from those of the vertebrate isoforms (Zlotkin, 1999).

Many animal toxins isolated from spider, scorpion, conus, and snake are found to selectively act on VGSCs (Olamendi-Portugal *et al.*, 2002). To date, more than 30 sodium channel toxins have been purified and well characterized from venoms of various species. Spider venoms contain many peptide toxins that target voltage-gated sodium channels. These spider toxins exhibit limited sequence identity with those of other origins, such as marine animals, scorpions, and snakes, revealing that there is a perspective for searching for new valuable ligands to dissect various VGSCs. Furthermore, spider toxins have been shown to produce new insecticides and pharmaceuticals. Up to now, nine different receptor sites have been described on the α-subunit (Wang and Wang, 2003). Spider toxins mainly interact with three of them, corresponding to blocking channel pore [site 1, hainantoxin-I (HNTX-I) and huwentoxin-IV (HWTX-IV)], slowing channel inactivation (site 3, μ-agatoxins and δ-atracotoxins), and inhibiting channel activation (site 4, Magi 5 and ProTx I-II), respectively (Omecinsky *et al.*, 1996; Nicholson *et al.*, 1998; Middleton *et al.*, 2002; Peng *et al.*, 2002; Corzo

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Foundation item: National Natural Science Foundation of China (Grant no. 30500146); Key State Basic Research Development Program of China ("973" Program) (Grant no. 2006CB708508)

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Received: 2008-09-01; Accepted: 2008-12-18

et al., 2003; Li *et al.*, 2003).

Huwentoxin-III (HWTX-III) is a 33-amino-acid peptide isolated and purified from the crude venom of *Ornithoctonus huwena* (Wang *et al.*) (Huang *et al.*, 2003). Our previous experiments indicated that the toxin can reversibly paralyze cockroaches for several hours with an ED_{50} value of 50 ± 30 nmol/g (Huang *et al.*, 2003).

In this study, we use patch clamp methods to characterize the actions of HWTX-III on voltage-gated ionic channels expressed in both cockroach dorsal unpaired median (DUM) neurons and rat dorsal root ganglion (DRG) neurons. The toxin had no effect on voltage-gated potassium channels (VGPCs) (data not shown) and on VGSCs in adult rat DRG neurons (data not shown), but selectively inhibited VGSCs on cockroach DUM neurons. The novel function of HWTX-III provides theoretical foundations for exploiting novel and safe insecticides.

2 MATERIALS AND METHODS

2.1 Venom and animals

The venom from the female adult *S. huwena* was collected as described in our laboratory earlier (Shu and Liang, 1999). Adult Sprague-Dawley rats and cockroach *Periplaneta americana* were from our laboratory stock.

2.2 Toxins and reagents

HWTX-III was purified by means of ion-exchange and reverse-phase high performance liquid chromatography (RP-HPLC) from the venom of the Chinese bird spider *S. huwena* as previously described (Huang *et al.*, 2003). The purity of HWTX-III was over 98%, as assessed by RP-HPLC and mass spectrometry analysis. Tetrodotoxin, trypsin (type III), collagenase (type IA), Dulbecco's modified Eagle's medium, and EGTA were purchased from Sigma Chemical Co., USA. All reagents were of analytical grade.

2.3 Cell preparation

Cockroach dorsal unpaired median neurons were acutely dissociated from the terminal abdominal ganglion (TAG) of the cockroach *P. americana* and maintained in a short-term primary culture. Briefly, mature cockroaches were killed in 75% alcohol and washed in saline solutions containing the following substances (in mmol/L): NaCl 200, KCl 3.1, $MgCl_2$ 4, HEPES 10, and sucrose 50 at pH 7.4. After wiped off the enteron, the TAG was removed quickly, and was incubated at room temperature (20 – 25℃) in enzyme solution for 5 min containing the following substances (in mmol/L): NaCl 200, KCl 3.1, $MgCl_2$ 4, HEPES 10, and sucrose 50, including trypsin 0.3% and collagenase IV 0.5% at pH 7.4. Their enzymatic digestion was stopped in 35 mm culture dishes containing NaCl (200 mmol/L), KCl (3.1 mmol/L), $MgCl_2$ (4 mmol/L), HEPES (10 mmol/L), sucrose (50 mmol/L), fetal bovine serum (5%), and penicillin (50 IU/mL), and streptomycin (50 μg/mL) at pH 6.8. After the cells in the ganglia were gently dispersed using suction tube, they were incubated in CO_2 incubator (5% CO_2, 28℃) for 20 min to 2 h before patch clamp experiment.

Rat dorsal root ganglion neurons were acutely dissociated and maintained in a short-term primary culture according to the procedures adapted from Xiao *et al.* (2005). Briefly, 30-day adult Sprague-Dawley rats of either sex were killed by CO_2 anesthesia followed by decapitation, and the dorsal root ganglia were removed quickly from the spinal cord and then transferred into Dulbecco's modified eagle's medium (DMEM) containing trypsin (0.5 g/L, type III) and collagenase (1.0 g/L, type IA) to incubate at 34℃ for 30 min. Trypsin inhibitor (1.5 g/L, type II-S) was used to terminate enzyme treatment. The isolated dorsal root ganglion cells were suspended in essential DMEM medium supplemented with 10% newborn calf serum and $NaHCO_3$ (3.6 g/L). The cells were plated in 35 mm culture dishes (Corning, Sigma) and incubated in CO_2 incubator (5% CO_2, 37℃). Cells were incubated for 3 – 4 h before the patch clamp experiment.

2.4 Solution preparation

Na^+ currents from cockroach DUM neurons were recorded using the external bathing solution containing the following substances (in mmol/L): NaCl 100, KCl 4, D-glucose 10, $CaCl_2$ 2, HEPES 10, choline-Cl 50, tetraethylammonium chloride (TEA-Cl) 20, 4-AP 1, and $CdCl_2 \cdot 2.5H_2O$ 0.02 at pH 6.8; and using the internal solution containing the following substances (in mmol/L): NaCl 100, KCl 4, D-glucose 10, $CaCl_2$ 2, HEPES 10, choline-Cl 50, tetraethylammonium chloride (TEA-Cl) 20, 4-AP 1, and $CdCl_2 \cdot 2.5H_2O$ 0.02 at pH 6.8.

Na^+ currents from adult rat DRG neurons were recorded using the external bathing solution containing the following substances (in mmol/L): NaCl 30, CsCl 5, D-glucose 25, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and TEA · Cl 90 at pH 7.4; and using the internal solution containing the following (in mmol/L): CsF 105, NaCl 35, HEPES 10, and EGTA 10 at pH 7.4 with CsOH.

2.5 Electrophysiological recording

Currents were recorded from experimental cells using an EPC-9 patch clamp amplifier (HEKA Electronics, Germany) under whole-cell patch clamp configuration at room temperature (22 – 25℃). Recording pipettes (2 – 3 μm diameter) were made from borosilicate glass capillary tubing and their resistances were 1.0 – 3.0 MΩ when filled with internal solution. The needed concentrations of toxin dissolved in external solution were applied onto the surface of experimental cells by low-pressure injection with a microinjector (IM-5B, Narishige). During the whole experiment the resistance was kept near 5 MΩ and compensated 50% – 70%, and linear capacitive and leakage currents were digitally subtracted by using the P/4 protocol. Experimental data were acquired and analyzed by the program pulse + pulsefit8.0 (HEKA Electronics, Germany) and Sigmaplot9.0 (Sigma, USA). All data are presented as mean ± standard error and n is the number of independent experiments. The fitted curves of both concentration-dependent inhibition [inhibition (%)] and steady-state sodium channels inactivation ($I/I_{\max}$) were obtained by using the following form of the Boltzmann equation:

$$\text{Inhibition}\ (\%) = 100/[1+\exp(C-\mathrm{IC}_{50})/k] \quad (1)$$

$$I/I_{max} = 1/[1+\exp(V-V_{1/2})/k] \quad (2)$$

In Eq. (1), IC_{50} is the concentration of toxin at half-maximal inhibition, k is the slope factor, while C is the toxin concentration. In Eq. (2), $V_{1/2}$ is the voltage at half-inactivation, k is the slope factor, while V is the test voltage.

3 RESULTS

3.1 Purification and sequence similarity

HWTX-III was purified by a combined use of ion-exchange HPLC and reverse-phase HPLC. The purity of huwentoxin-III was over 98%, as judged by RP-HPLC and mass spectrometry analysis (data not shown). The analysis of BLAST search (www.ncbi.nlm.nih.gov/blast) and amino acid sequence alignment (using CLUSTAL M program) indicated that HWTX-III has less than 40% sequence identity with HNTX-I, HNTX-IV, JZTX-III which inhibited tetrodotoxin-sensitive (TTX-S) sodium channels on neurons (Li *et al.*, 2003; Li *et al.*, 2004; Xiao *et al.*, 2004), but shares 50% identities with SHL-I which agglutinated human A, B and O-type erythrocytes (Liang and Pan, 1995). The six cysteine residues in huwentoxin-III were also well conserved at similar positions among these toxins (Fig. 1).

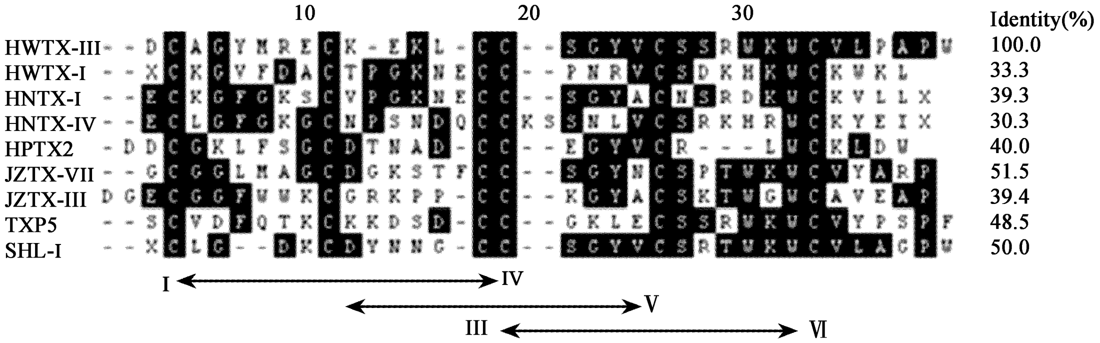
10
20
30
Identity(%)
HWTX-III - - D C A G Y M R E C K - E K L - C C - - S G Y V C S S R W K W C V L P A P W 100.0
HWTX-I - - X C K G V F D A C T P G K N E C C - - P N R V C S D K H K W C K W K L 33.3
HNTX-I - - E C K G F G K S C V P G K N E C C - - S G Y A C N S R D K W C K V L L X 39.3
HNTX-IV - - E C L G F G K G C N P S N D Q C C K S S N L V C S R K H R W C K Y E I X 30.3
HPTX2 - D D C G K L F S G C D T N A D - C C - - E G Y V C R - - - L W C K L D W 40.0
JZTX-VII - - G C G G L M A G C D G K S T F C C - - S G Y N C S P T W K W C V Y A R P 51.5
JZTX-III D G E C G G F W W K C G R K P P - C C - - K G Y A C S K T W G W C A V E A P 39.4
TXP5 - - S C V D F Q T K C K K D S D - C C - - G K L E C S S R W K W C V Y P S P F 48.5
SHL-I - - X C L G - - D K C D Y N N G - C C - - S G Y V C S R T W K W C V L A C P W 50.0
I
IV
V
III
VI

Fig. 1 Comparison of amino acid sequence of HWTX-III with HWTX-I, HNTX-I, HNTX-IV, HPTX2, JZTX-III, JZTX-VII, TXP5 and SHL-I

HWTX-III, HWTX-I and SHL-I were isolated from *Ornithoctonus huwena*, HNTX-I and HNTX-IV were isolated from *Ornithoctonus hainana*, JZTX-VII and JZTX-III were isolated from *Chilobrachys jingzhao*, TXP5 was isolated from the Mexican red knee tarantula and HpTX2 was isolated from *Heteropoda venatoria* spider. The identical residues are shaded in black. The conserved disulfide bridge pattern of these toxins is indicated under their sequences.

3.2 Effects of HWTX-III on voltage-gated sodium channels

Multiple ion channels express in both vertebrate dorsal root ganglion neurons and insect dorsal unpaired median neurons and underlie spontaneous electrical activity. It is widely accepted that tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels co-express in adult rat dorsal root ganglion neurons (Xiao *et al.*, 2005), whereas all the VGSCs in cockroach dorsal unpaired median neurons are tetrodotoxin-sensitive sodium channels (Lapied *et al.*, 2001). The actions of HWTX-III on VGSCs were characterized in *P. americana* DUM neurons and rat dorsal root ganglion neurons by using whole-cell patch clamp technique. After the whole-cell recording configuration was established, the experimental cells were held at – 80 mV for at least 5 min to allow adequate equilibration between the micropipette solution and the cell interior, and then the current traces were evoked using a 50 ms step depolarization to – 10 mV every second. As shown in Fig. 2, huwentoxin-III exhibited evident effects on inward sodium currents from cockroach dorsal unpaired median neurons, 100 nmol/L HWTX-III reduced the control peak amplitude to a maximum effect by 28. 50% ± 6. 9% (Fig. 2, n = 5). The rapid inhibition was in a dose-dependent manner, and their IC_{50} values were assessed to be 1. 106 μmol/L (Fig. 2, n = 5). Reduced by the neurotoxin, the shape of currents was similar to that of control, and the same results were found at other concentrations such as 50 nmol/L and 1 μmol/L.

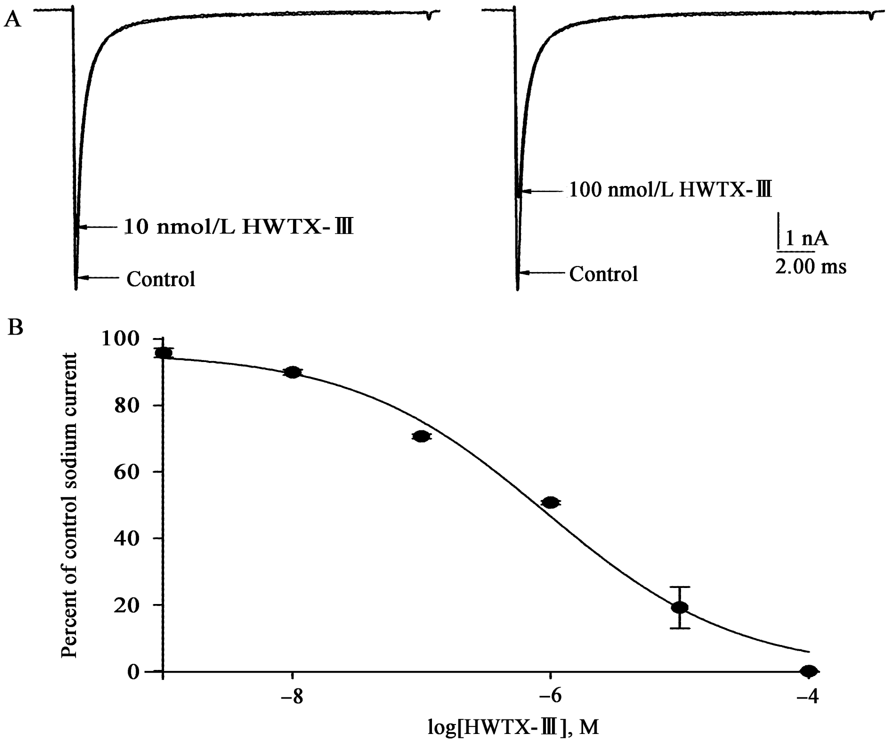

Fig. 2 Effects of HWTX-III on sodium currents in insect DUM neurons

All current traces were elicited by depolarizing the cell from a holding potential of − 80 mV to − 10 mV. The duration of the test pulse was 20 ms. A: 10 nmol/L, 100 nmol/L HWTX-III caused a significant decrease in peak current amplitude. B: The concentration-dependent inhibition of sodium currents in insects. Every point (mean ± *SE*) comes from 5 − 8 separated experimental cells. These points were fitted according to Boltzmann equation: Inhibition (%) = $100/[1 + \exp(C - IC_{50})k]$, in which IC_{50} was the concentration of HWTX-III at half-maximal inhibition, *k* was the slope factor, and *C* was the concentration of HWTX-III.

The effects of HWTX-III on the current-voltage relationship are illustrated in Fig. 3. After 1 μmol/L HWTX-III treatment for 1 min, there were no changes in both the threshold of activation and the active voltage of peak inward currents, and there was either no shift of the membrane reverse potential. HWTX-III shows no effect on the activation and inactivation kinetics of TTX-S VGSCs. Its action on TTX-S current is very similar to that of TTX (Wicher and Penzlin, 1998). Thus we presume it is reasonable to take HWTX-III as a site I toxin. To further assess the profound actions of HWTX-III on insect sodium channels, the effect of the toxin on steady-state inactivation was also investigated by using a standard two-pulse protocol as detailed in legend of Fig. 4. The results showed the voltage dependence of inactivation of VGSCs under the control conditions and in the presence of HWTX-III. They were almost completely overlapping, implying that HWTX-III did not affect the steady-state inactivation kinetics of the VGSC currents. The potential of the half-inactivation ($V_{1/2}$) and slope factor (k) were − 55. 72 ± 0. 38 mV and − 5. 43 ± 0. 46, respectively for the control conditions and − 55. 48 ± 0. 45 mV and − 4. 67 ± 0. 35, respectively in the presence of 5 μmol/L HWTX-III. The absence of any effect of HWTX-III on the voltage dependence of steady-state inactivation of VGSCs suggests that HWTX-III does not interact with VGSCs that are in the inactivated state.

No effect was detected on sodium channels from adult rat dorsal root ganglion neurons (data not shown).

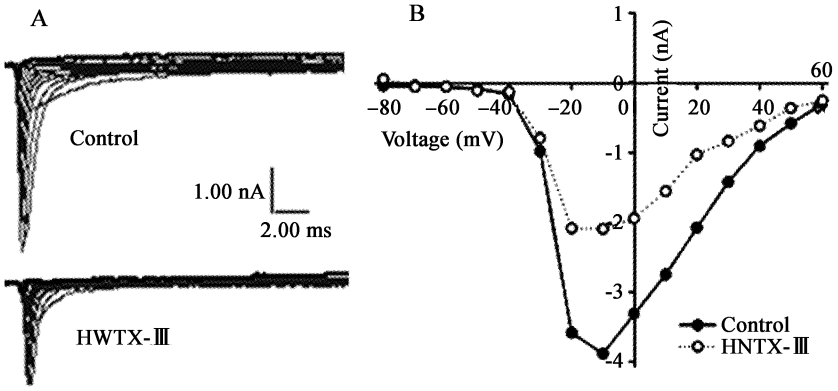

Fig. 3 Effects of HWTX-III on the current-voltage relationships of sodium channels

The current traces were induced by 20 ms depolarizing steps to various potentials from a holding potential of -80 mV. Test potentials ranged from -80 mV to $+40$ mV, with an increment of $+10$ mV. The I-V curves showed the relationships between current traces and potentials before and after adding 1 μmol/L HWTX-III in neurons (A). In B, the date points obtained from five separated experimental cells are shown as mean ± *SE*.

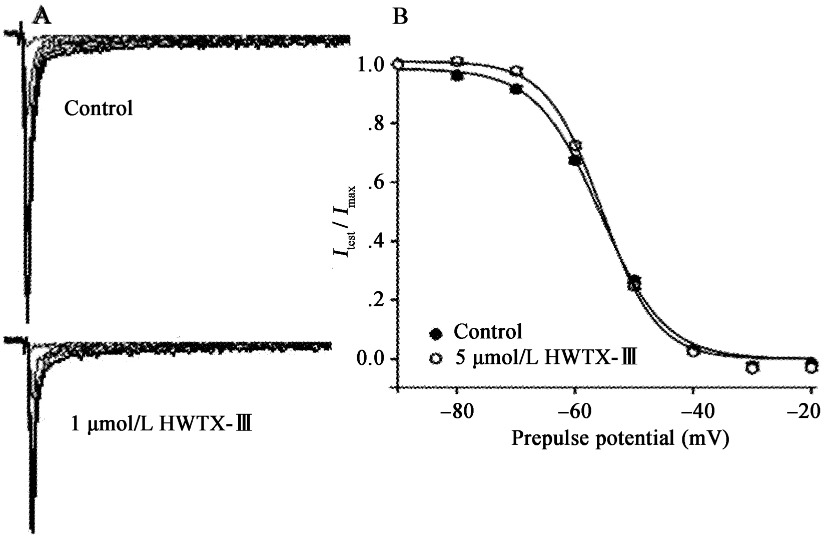

Fig. 4 Effects of HWTX-III on the steady-state inactivation of sodium channels

Sodium currents were induced by a 50 ms depolarizing potential of -10 mV from various prepulse potentials for 1 s ranging from -90 mV to -20 mV with an increment of $+10$ mV. A: Typical current traces were obtained following a 50 ms depolarization to -10 mV from prepulse potential of -90, -60, -50 and -40 mV; B: The data dots shown as the ratio of I_{test} to I_{max} were fitted according to the Boltzmann equation: $I_{test}/I_{max} = 1/[1+\exp(V-V_{1/2})/k]$, where V was the prepulse potential, $V_{1/2}$ was the voltage at which I was 0.5 I_{max}, and k was the slope factor. After the treatment of HWTX-III at 5 μmol/L, the $V_{1/2}$ values were not significantly shifted.

3.3 Effects of HWTX-III on voltage-gated potassium channels (VGPCs)

In order to demonstrate the selectivity of huwentoxin-III toward ion channels, we further tested the effect of huwentoxin-III on potassium channels. Delayed rectifier potassium current traces were evoked by a 300 ms depolarization to 30 mV from a holding potential of -90 mV. However, huwentoxin-III failure in changing the ion currents was observed (data not shown).

4 DISCUSSION

In the present study, we characterized the action of HWTX-III on voltage-gated Na^+ and K^+ channels in *P. americana* DUM neurons and rat DRG neurons by using whole-cell voltage clamp technique. It was found that the toxin showed no effect on VGPCs from both cockroach DUM neurons and rat DRG neurons (data not shown). However, huwentoxin-III obviously depressed the amplitude of sodium currents on cockroach DUM neurons. When the membrane potential was held at -80 mV, lower concentration of HWTX-III reduced peak sodium current amplitude. The reduction of current amplitude was in a concentration-dependent fashion with the IC_{50} value of 1.106 μmol/L. The *I/V* spectrum showed that HWTX-III did not shift the threshold of activation voltage and the membrane reversal potential, which indicated that it did not change the ion selectivity of channels. Furthermore, our patch clamp experiments showed that HWTX-III failed to target VGSCs on adult DRG neurons (data not shown), indicating it is a specific insect toxin. Although HWTX-III has low sequence similarity with HNTX-I, both of them could block insect neuronal VGSCs by interacting with receptor site 1 through a mechanism quite similar to that of TTX without affecting the activation and inactivation kinetics (Li *et al.*, 2003). Thus, we infer that HWTX-III belongs to depressant insect toxins. Furthermore, HWTX-III did not cause an evident shift of the steady-state inactivation cure, which indicated this mechanism of HWTX-III is different from that of other depressant spider toxins previously studied such as HWTX-I (Wang *et al.*, 2007), HNTX-V (Xiao and Liang, 2003b) and HNTX-III (Xiao and Liang, 2003a).

In summary, among all the reported spider toxins, huwentoxin-III is a new class of depressant spider toxins specifically affecting insect VGSCs. Our results have also proved that it is a class of novel and useful ligands for investigating the multiple molecular forms of VGSCs in insects and might be a useful biopesticide because of its specificity and insect-toxic effects. The selectivity towards ion channels of HNTX-III also suggests that it is harmless to other organisms, and thus it would be no danger if it entered the food chain. The discoveries and investigations of huwentoxin-III open new perspectives in developing new insecticidal compounds.

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虎纹捕鸟蛛毒素虎纹毒素-III 对美洲蜚蠊神经细胞电压门控离子通道的影响

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摘要： HWTX-III 是从中国虎纹捕鸟蛛 *Ornithoctonus huwena* 粗毒中分离纯化到的一种昆虫神经多肽。通过应用全细胞膜片钳技术研究了 HWTX-III 对美洲蜚蠊 *Periplaneta americana* 神经细胞电压门控离子通道的影响。发现 HWTX-III 特异性地抑制美洲蜚蠊背侧不成对中间(dorsal unpaired median, DUM)神经细胞的电压门控钠通道($IC_{50} \approx 1.106$ μmol/L)，而对电压门控钾通道没有明显的影响。HWTX-III 通过一种新型的不同于其他蜘蛛毒素的机制抑制昆虫电压门控钠通道，它不影响通道的激活与失活动力学，也不明显地漂移稳态失活曲线。HWTX-III 对昆虫神经细胞电压门控钠通道的特异性与新型作用机制为研究电压门控钠通道分子结构的多样性以及开发新的安全的杀虫剂提供有用的工具。

关键词： 虎纹捕鸟蛛；虎纹毒素-III；美洲蜚蠊；背侧不成对中间神经细胞；钠离子通道；全细胞膜片钳技术

中图分类号： Q966 **文献标识码：** A **文章编号：** 0454-6296(2009)02-126-07

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